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Heat treatment of milk protein concentrates affects enzymatic coagulation properties

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ABSTRACT

Dairy ingredients with highly concentrated protein contents are high added value products with expanding market. The manufacture of such ingredients includes a succession of unit operations of which heat treatment is a key step to guarantee the microbial safety, that induces major changes in protein structures and thus ingredients functionalities. However, due to an incomplete understanding of phenomena taking place at high protein concentrations, shedding light on their mechanisms is a scientific challenge as well as an industrial need. In this study, the influence of heat treatment (74 °C/ 30 s) of highly concentrated milk protein systems (up to 20 % w/w) on protein denaturation/aggregation and enzymatic coagulation properties was studied using an original semi–industrial approach. 10 % w/w protein solutions constituted with whey protein and casein micelles at milk ratio, standardized in osmosed water or ultrafiltration permeate were used. These protein solutions were processed in different ways prior the manufacture of powders: heat treatment of the 10 % w/w protein solution before vacuum evaporation, heat treatment of the 20 % w/w protein solution after vacuum concentration, two consecutive heat treatments before and after vacuum evaporation. A fourth powder was prepared from unheated 10 % w/w protein solution. An increase in protein concentration led to a higher heat-induced protein denaturation. This phenomenon was reduced when increasing the lactose content. The effect of heat treatment on the extent of protein denaturation was not cumulative. At high protein concentration, interactions between κ-casein and whey protein were modified compared to milk, as mainly micelle-bound aggregates were formed at pH about 6.7. This phenomenon was enhanced at low ionic strength and lactose content. Our study showed that the enzymatic coagulation properties of reconstituted protein powders could be correlated with their physico–chemical compositions. An increase in protein denaturation disrupted the gel reorganization and led to the formation of weaker gels but did not interfere on the micelles aggregation phase and the early gelation. On the contrary, an increase in ionic strength and lactose content led to higher gel time.

1. Introduction

Dairy protein ingredients are high added value products with expanding market. They are known to have specific properties adapted for a multitude of applications (cheese, infant formulae…). In particular, dairy powders with highly concentrated protein content have been gaining more and more attention, influenced by the thriving market of infant formulae and specialized nutrition products for the elderly or athletes (Lagrange, Whitsett, & Burris, 2015). Understanding the behavior of such complex-high protein content ingredients when applied to a food matrix is therefore a paramount value for food industries in order to obtain final products with the desired characteristics. Dairy protein ingredients in dried form are obtained through a succession of unit operations where heat treatment results in a number of changes in protein structures, caused by the denaturation and aggregation of whey proteins and their interactions with casein micelles

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(Singh & Creamer, 1992; Murphy, Tobin, Roos, & Fenelon, 2013; Singh & Havea, 2003; Walstra & Jenness, 1984). Thermal denaturation and aggregation of whey proteins, especially β-Lactoglobulin (β-Lg), have been largely studied. The denaturation takes place in two steps, initiated by the native proteins unfolding during heating. Then, the unfolded proteins aggregate *via* intermolecular disulphide bonds and hydrophobic interactions (Havea, Singh, & Creamer, 2001; Hong & Creamer, 2002; Wijayanti, Bansal, & Deeth, 2014; Zúñiga, Tolkach, Kulozik, & Aguilera, 2010). The lactose concentration is known to affect the denaturation. An increase in denaturation temperature of β-Lg with increasing lactose concentration has been reported (de Wit & Klarenbeek, 1984; Plock, Spiegel, & Kessler, 1998). These phenomena have been largely investigated at relatively low protein concentration up to 10 % w/w (Boye, Alli, & Ismail, 1997; de Wit, 2009; Nicolai, Britten, & Schmitt, 2011) and it has been showed that they were dependent on the ionic strength, the pH and the heating temperature. Only a handful of studies were focused on protein content up to 20 % w/w (Dissanayake, Ramchandran, Donkor, & Vasiljevic, 2013; Nielsen, Singh, & Latham, 1996). Whey proteins, when denatured, have been shown to interact with κ-casein on the surface of casein micelles (Anema & Li, 2003; Donato & Guyomarc'h, 2009; Creamer & Matheson, 1980; Mohammad & Fox, 1987). The main association is reckoned to occur between β–Lg and κ-casein and involves both disulphide and hydrophobic interactions (Singh & Fox, 1987; Smits & Van Brouwershaven, 1980). The extent of association of denatured whey proteins with casein micelles is dependent on the pH and the level of soluble calcium and phosphate. Oldfield, Singh, & Taylor (1998) proposed a mechanism for β-Lg denaturation and its association with casein micelles in milk systems. They suggested that at least three possible species of denatured β-Lg could interact with micelles and the relative rates of these interactions depended on the temperature and the heating rate. The effects of denaturation of whey protein and its association with casein micelles on rennet coagulation properties have been studied (Britten & Giroux, 2022; Dalgleish, 1990; Donato & Guyomarc'h, 2009; Waungana, Singh, & Bennett, 1996). Heat treatment of milk results in longer rennet coagulation times and reduced strength of rennet gels (Banks, 1990; Dalgleish, 1992; Morrissey, 1969). The influence of denatured whey proteins on the renneting properties and the characteristics of cheese has also been studied (Lebeuf, Lacroix, & Paquin, 1998; Mead & Roupas, 2001). The addition of heat-denatured whey proteins to milk decreased the strength and the contraction capacity of rennet-induced gels, resulting in cheese with increased moisture content (Hinrichs, 2001). These changes were attributed to the strong water-binding capacities of whey protein aggregates. Therefore, the addition of denatured whey proteins in cheese milk is a way to level up the protein content in cheese, with the final objective of increasing the cheese yield (Jensen & Stapelfeldt, 1993) with simultaneous nutritional interest (Ismail, Ammar, & El-metwally, 2011). Hence, the impact of heat treatment on protein systems and their rennet coagulation properties are abundantly documented for relatively low concentrated systems such as milk. However, to the best of our knowledge, there is no study dealing with protein denaturation and aggregation in highly concentrated systems containing both whey proteins and casein micelles. It is not possible from the current knowledge to fully understand the impact of heat treatment according to the concentration and composition of the protein base on the protein structures and their ability for rennet coagulation.

Thus, this study aims to investigate the influence of heat treatment of highly concentrated milk protein systems on protein denaturation/aggregation process and enzymatic coagulation properties. The stakes of this study are double: i) applicative since it can be used to propose tools for quality-functionality control in applications, such as cheese making and ii) cognitive because, at this state, there is very little knowledge on highly concentrated protein products.

2. Materials and methods

2.1. Preparation of the standardized protein solutions

Whey protein concentrate (27 % dry matter w/w, 95 % protein on dry matter) and native phosphocaseinate concentrate (13 % dry matter w/w, 87 % protein on dry matter) were provided by two different dairy companies (France). Both were standardized down to 10 % w/w of protein using osmosed water or fresh milk ultrafiltration permeate (Industrial source, France). They were mixed in order to obtain standardized solutions with casein to whey protein ratio of 80:20 as in bovine milk. For each experimentation, a batch of 500 L of protein solutions was prepared.

2.2. Fabrication of protein powders

Both standardized protein solutions were processed up to the manufacture of dairy powders. Three process schemes with different positions of heat treatment were applied: heat treatment of the 10 % w/w protein solution before vacuum evaporation (HT 10 %), heat treatment of the 20 % w/w protein solution after vacuum concentration (HT 20 %), two consecutive heat treatments before and after vacuum evaporation (2 HT). A fourth powder was prepared from unheated 10 % w/w protein solution (Control) (Fig. 1).

2.2.1. Continuous heat treatment

Heat treatment of standardized protein solutions were conducted in a tubular exchanger (Tetra Pak, Lund, Sweden) at 74 °C for 30 s and then rapidly cooled down to 20 °C using a second tubular exchanger. The heating tubular exchanger was made up of 11 tubes (diameter = 16 mm, length = 3 m). Temperature probes were placed at the end of each tube in order to monitor the changes in temperature through the exchanger. The flow rate was set at 120 $L.h^{-1}$.

2.2.2. Vacuum evaporation

Vacuum evaporation was carried out using a two-stage falling film vacuum evaporator (GEA, Saint-Quentin-en-Yvelines, France, France) with an evaporation capacity of 180 $kg.h^{-1}$. The inlet flow rate was 250 $L.h^{-1}$. The product temperature was 55 °C at the outlet of the first stage and 37 °C at the outlet of the evaporator.

2.2.3. Spray drying

Spray drying was carried out using a three-stage drying pilot (GEA, Niro atomizer, Saint-Quentin-en-Yvelines, France). The inlet temperature of the concentrated protein solution was 40 °C and the feeding flow rate was 100 $L.h^{-1}$. The atomizer was equipped with a pressure nozzle (0.63 mm of diameter orifice, n°72) providing a 55° spray angle. Inlet air humidity was adjusted by a dehumidifier (Munters, Sollentuna, Sweden) at 1 $g.kg^{-1}$ of water in dry air. The inlet and outlet air temperature were 218–223 °C and 81–84 °C, respectively, in order to obtain powders of water activity (a_w) = 0.2. After drying, powders were packed into aluminium tins and stored at 13 °C before further analysis.

2.3. Physicochemical characterization

Before physicochemical characterization, powders were reconstituted at 50 $g.kg^{-1}$ protein content. Powders were solubilized in osmosed water at room temperature, for 48 h using a stirring device (IKA Eurostar 20, Staufen im Breisgau, Germany) operating at 600 rpm.

2.3.1. Protein content

The total nitrogen (TN) and the non-protein nitrogen (NPN) contents were determined using the Kjeldahl method (ISO 8968–1, International Standard Organisation, 2014). For the NPN analysis, protein solutions were first precipitated using 15 % w/v trichloroacetic acid (PanReac, Lyon, France) and filtered through a 8 μm Whatman® filter (Merck,

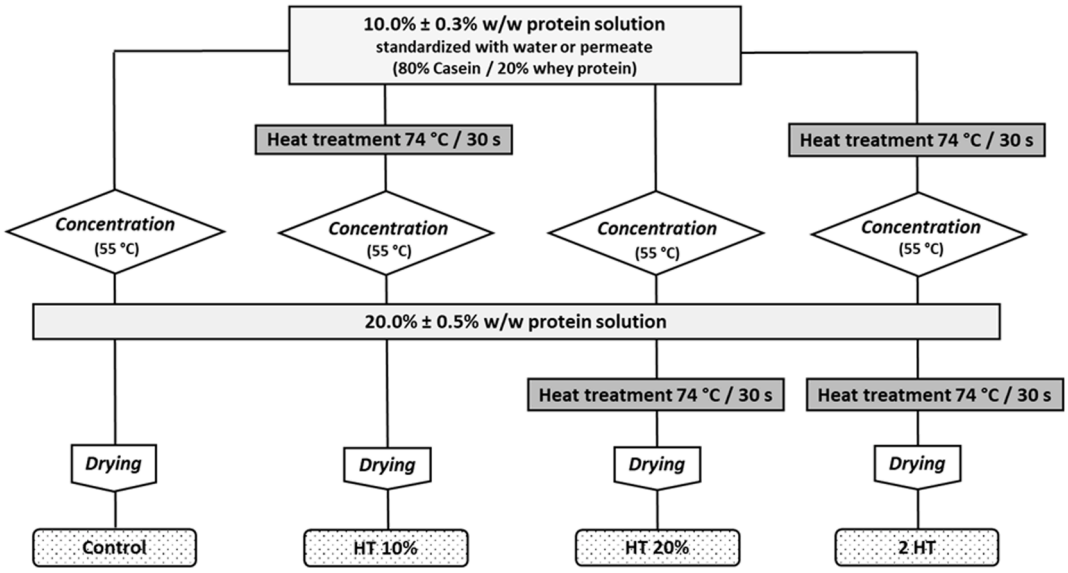

Fig. 1. Process schemes for protein powder production. HT 10 %: heat treatment of the 10 % w/w protein solution before vacuum evaporation; HT 20 %: heat treatment of the 20 % w/w protein solution after vacuum concentration; 2 HT: two consecutive heat treatments before and after vacuum evaporation.

Darmstadt, Germany). The protein content was calculated according to Equation (1) with 6.38 as the nitrogen to protein conversion factor (Schuck, Dolivet, & Jeantet, 2012).

$$\text{Protein content} = (\text{TN} \times 6.38) - (\text{NPN} \times 6.38) \quad (1)$$

2.3.2. Percentage of native whey proteins over total whey proteins

Protein solutions were first acidified to pH 4.6 with 10 % w/v acetic acid (VWR International, Fontenay-sous-Bois, France) and then filtered through a 2.5 µm Whatman® filter (Merck, Darmstadt, Germany). Non–casein nitrogen (NCN) in the filtrate was measured using Kjeldahl method (ISO 8968–1, International Standard Organisation, 2014). The percentage of native whey proteins over total whey proteins was calculated according to Equation (2) (Schuck et al., 2012).

$$\frac{\text{Native whey protein}}{\text{Total whey protein}}(\%) = \frac{(\text{NCN} \times 6.38) - (\text{NPN} \times 6,38)}{\left(\text{NCN}_{\text{initial protein solution}} \times 6.38\right) - \left(\text{NPN}_{\text{initial protein solution}} \times 6.38\right)} \times 100 \quad (2)$$

2.3.3. Co-aggregation whey protein/casein micelles

Experiments were based on the methods of Noh & Richardson (1989), Pesic et al. (2012) and Vasbinder, Alting, & De Kruif (2003). Indeed, protein fractionation was conducted using a combination of acetic acid and rennet precipitation. Acetic acid precipitation separates native whey proteins from aggregated ones. Rennet precipitation separates whey protein micellar aggregates from soluble whey proteins (native or in aggregates) and then allows the determination of native whey protein, whey protein soluble aggregates and micelle-bound whey protein aggregates content. The β–Lg and α-lactalbumin (α-lac) concentrations after rennet and acid fractionations were determined by high performance liquid chromatography (HPLC) coupled with a UV detector. The chromatographic system was an Alliance e2695 Separation Module (Waters, Milford, MA, USA), with a ACE C4 300 Å, 5 µm, 4.6 mm × 250 mm separation column. The two mobile phases were 0.1 % v/v of trifluoroacetic acid (99 % purity, Acros Organics, Thermo Fischer Scientific, Waltham, MA, USA) in ultrapure water as solvent A and 0.1 % v/v trifluoroacetic acid in acetonitrile (HPLC grade, Thermo Fisher Scientific, Waltham, MA, USA) as solvent B. Twenty microliter of each sample (kept at 10 °C) was injected in the column maintained at 30 °C. The separation was performed at a flow rate of 1 mL.min^{-1} with a detection wavelength at 220 nm. The gradient profile was 0–40 min linear gradient from 30 % B to 50 % B; 40–42 min linear gradient from 50 % B to 100 % B; 42–43 min isocratic elution 100 % B; 43–46 min linear gradient from 100 % B to 30 % B, followed by a 5 min isocratic elution at the initial conditions. Analyses were conducted twice for each sample and standard. Calibration standards with concentrations ranging from 0.1875 to 1.5 g.L^{-1} and from 0.0625 to 0.5 g.L^{-1} were prepared by dissolving β-Lg (88.85 % purity, BioPure industrial powder, Davisco, Foods International, Inc., La Sueur, MN, USA) and α-lac powders (>90 % purity, Sigma-Aldrich, Saint Louis, MO, USA) in HPLC buffer (8 M urea, 0.1 M Bis-Tris pH 8.44 mM sodium citrate and 0.3 % v/v dithiothreitol).

The native β-Lg and α-lac concentrations were calculated by averaging the measured chromatographic areas and converting each area into concentration by using the calibration curves.

2.3.4. Free lactose content

The protein solution was centrifuged at 109 000 g for 90 min at 30 °C (Sorvall, Discovery 90SE, Hitachi, USA). The supernatant was then ultrafiltrated with a VivaSpin membrane (molecular weight cut-off of 10 kDa, Sartorius, Göttingen, Germany) at 1800 g for 30 min (Centrifuge 5810 R, Eppendorf, Hamburg, Germany) to obtain its soluble phase. Lactose of the soluble phase was separated and detected by HPLC using a Dionex system (Dionex, Germering, Germany), equipped with a column oven heated at 60 °C, coupled to a differential refractometer (model RI 2031 plus, Jasco). The stationary phase was a 300 × 6.5 mm column containing an ion exchange resin Aminex A-6 (Biorad, Saint Louis, MO, USA) (Norwood et al., 2017). The elution was carried out using a 0.4 mL.min^{-1} using flow rate with 5 mM H_2SO_4 solution. The analysis was conducted in triplicate.

2.3.5. Total ash and calcium contents

Five grams of the protein solution was placed in a porcelain crucible and heated in a muffle furnace (Nabertherm, Lilienthal, Germany) at 550 °C for 5 h until a white or grey ash residue was obtained. The ash content was determined in triplicate following the method described by Schuck et al. (2012). The ash content was calculated according to Equation (3) (Schuck et al., 2012).

$$\text{Ash content}\,(\text{g.100g-1}) = \frac{(Mass_{crucible+ashresidues} - Mass_{crucible})}{(Mass_{crucible+proteinsolution} - Mass_{crucible})} \times 100 \quad (3)$$

The ash residues were dissolved and diluted with 2 % v/v HNO_3 (Thermo fisher scientific, Waltham, MA, USA). The calcium content was measured using an inductively coupled plasma-optical emission spectrometer (ICP-OES) (ICAP 7200, Thermo fisher scientific, Courtaboeuf, France). Argon was used as operating gas. Calibration curve was obtained using calcium standards from Reagecon (Shannon, Ireland).

2.4. Coagulation measurements

2.4.1. Renneting

Coagulation measurements were also carried out on reconstituted solutions at 50 $g.kg^{-1}$ protein content. Sodium azide (Sigma-Aldrich, Saint Louis, MO, USA) was added at a final concentration of 0.2 $g.L^{-1}$. The pH of the solutions was adjusted to 6.60 ± 0.03 the day before the coagulation experiments, using 1 M HCl (PanReac, Lyon, France). Before renneting, samples were kept at 30 °C for 30 min for equilibrium. The pH was checked and re–adjusted to 6.60 if needed. Three hundred microliters of rennet (CHY-MAX Supreme, 10 $IMCU.mL^{-1}$, Chr. Hansen, Horsholm, Denmark), were added to 50 mL of protein solution and then gently stirred by hand.

2.4.2. Casein micelles aggregation

In order to measure kinetics of casein micelles aggregation, right after rennet addition, 18 mL of sample were quickly poured into disposable glass vats and then put into a Turbiscan LAB (Turbiscan Laboratory Formulation, Ramonville St. Agne, France) set at 30 °C. The increase in particles size due to the aggregation of hydrolyzed casein micelles was monitored using the increase in the backscattered signal. *Para*-micelles aggregation experiments and data analysis were performed following the methods of Bauland et al. (2020), Castillo, Payne, Hicks, Laencina, & López (2003), Payne & Castillo (2007). First, the backscattered signal increased due to an increase in particle size (*para*-micelles aggregation), then a transition phase occurred during which the signal remained constant (overlapping between aggregation and curd firming (Castillo et al., 2003) and finally the backscattered signal increased again. These authors assumed that aggregation was the dominant process between the inflection point and the beginning of the transition phase. The aggregation time was then defined as the crosslink point between abscissa axis and the tangent at the inflection point (Bauland et al., 2020). The backscattered signal intensity was recorded every 25 s for a total run duration of 25 min.

2.4.3. Rheological measurement of coagulation

Rennet coagulation kinetics were monitored using a DHR-2 rheometer (TA Instruments, Guyancourt, France) equipped with a Couette geometry (inner and outer radii = 28.0 and 30.4 mm) (Bauland et al., 2020) at a frequency of 0.1 Hz and 1.0 % strain. Just after rennet addition, 20 mL of solution was poured in the rheometer. Temperature in the system was kept at 30 °C using a Peltier thermal system. Storage modulus G' and loss modulus G'' were recorded for 60 min. As proposed by Giroux, Dupont, Villeneuve, & Britten (2020), gel time was defined as the time needed to reach a G' value of 1 Pa. The loss tangent (tan δ) was defined as the ratio of the viscous to the elastic modulus of the system, G''/G'. $G'_{60\ min}$ and tan $\delta_{60\ min}$ were the final values after 60 min of coagulation. The maximum rate of firming is the slope ($Pa.min^{-1}$) at the inflection point of the curve. The firming time is the difference between gel time and *para*-micelle aggregation time. Firming time could be interpreted as the time required for *para*-casein micelles to interact and reorganize spatially in order to initiate the formation of a protein network with viscoelastic properties.

2.5. Statistical analysis

Statistical analyses were conducted with the use of R (The R Foundation 2014). An ANOVA test was conducted after verifying that the residues of this model were normal with the Kolmogorov-Smirnov test ("lillie.test" from the "nortest" package). A post-hoc test ("LSD.test" of the "agricolae" package) was then conducted when the differences were significant ($p < 0.05$). Principal component analysis (PCA) was performed using the FactoMineR and Factoshiny packages (Lê, Josse, & Husson, 2008). The dataset was composed of 10 variables and 25 individuals (triplicate or quadruplicate of the eight studied samples). The variable "Micelle bound β-Lg / tot β-Lg denat" corresponds to the amount of denatured β-Lg bound to the casein micelles over the total amount of denatured β-Lg. Similarly, the variable "Micelle bound α-lac / tot α-lac denat" corresponds to the amount of denatured α-lac bound to the casein micelles over the total amount of denatured α-lac. The variable "% Denat" refers to the whey protein denaturation level. Values taken by the variable lactose/protein were the same for 10 % w/w protein samples and for 20 % w/w protein samples as the increase of lactose and protein contents induced by the vacuum evaporation step was identical.

3. Results and discussion

3.1. Composition of the standardized protein solutions

In all solutions, experimental protein contents (10.0 ± 0.5 and 20.0 ± 0.7 $g.100\,g^{-1}$) were conformed to the expected concentrations (10 and 20 %). The slight differences in protein content at the same targeted concentration were due to the replication of the process scheme for obtaining the standardized protein solution (Fig. 1). Ultrafiltration permeate is a dairy liquid stream at a low dry matter content (about 60 $g.kg^{-1}$) that contains mainly lactose (92 % of dry matter w/w) and minerals (7 % of dry matter w/w). Its mineral fraction is high in monovalent ions, such as potassium and chloride (about 1500 and 1100 $mg.kg^{-1}$, respectively) while it is low in calcium (about 280 $mg.kg^{-1}$) (Granger-Delacroix, 2020). This rough composition explained a higher free lactose content in protein solutions standardized with permeate compared to those standardized with water (6 to 9-fold) and a slightly higher level of ash. Besides, as shown in Table 1, the total calcium content was not significantly different between water- and permeate-standardized solutions. Finally, the higher amount of minerals in permeate-standardized solutions contributed to a higher ionic strength and a resulting lower pH (Tanguy, 2019) in solutions after vacuum evaporation (20 % w/w protein) compared to water–standardized solutions (Table 1).

3.2. Influence of protein concentration on the extent of heat-induced whey protein denaturation

The extent of whey protein denaturation (*i.e.* diminution of native whey protein content) was measured in the concentrated standardized protein solutions that had undergone the different process schemes: unheated solution (control), solutions heat–treated once at 10 % or 20 % protein concentration (HT 10 % and HT 20 %, respectively), solutions heat–treated twice (2 HT) (Fig. 2). The denaturation was reckoned to be induced exclusively by heat treatment. Indeed, it is generally accepted that during evaporation at temperatures between 50 and 63 °C (Gray, 1981; Oldfield, Taylor, & Singh, 2005; Singh & Creamer, 1991; Yu et al., 2021), there is no significant denaturation of whey protein. Whey protein denaturation remained marginal (>98 % present in native forms) for all unheated protein solutions whereas important decreases in native

Table 1
Physico-chemical composition of standardized protein solutions.

Process scheme	Sampling location	[Protein] (g.100 g^{-1}*)	pH	[Free lactose] (g.100 g^{-1}*)	[Ashes] (g.100 g^{-1}*)	[Ashes]/[Protein] (%)	[Ca] (g.100 g^{-1}*)	[Ca]/[protein] (%)
Protein solutions standardized with water								
Control	Unheated before concentration	$10.37^{e} \pm 0.02$	$6.78^{b} \pm 0.01$	$0.25^{e} \pm 0.01$	$0.93^{g} \pm 0.00$	$8.97^{e} \pm 0.01$	$0.31^{d} \pm 0.00$	$3.00^{abc} \pm 0.04$
HT 10 %	Before HT	$10.37^{e} \pm 0.02$	$6.77^{b} \pm 0.01$	$0.25^{e} \pm 0.01$	$0.93^{g} \pm 0.00$	$8.97^{e} \pm 0.01$	$0.32^{d} \pm 0.00$	$3.04^{abc} \pm 0.00$
HT 20 %	Before HT	$20.56^{b} \pm 0.03$	$6.68^{d} \pm 0.01$	$0.40^{d} \pm 0.01$	$1.65^{d} \pm 0.02$	$8.03^{f} \pm 0.08$	$0.57^{c} \pm 0.00$	$2.75^{d} \pm 0.01$
2 HT	Before 1st HT	$10.42^{e} \pm 0.02$	$6.77^{b} \pm 0.01$	$0.26^{e} \pm 0.01$	$0.98^{f} \pm 0.00$	$9.41^{d} \pm 0.03$	$0.31^{de} \pm 0.00$	$2.98^{bc} \pm 0.03$
	Before 2nd HT	$19.65^{d} \pm 0.02$	$6.70^{c} \pm 0.01$	$0.46^{d} \pm 0.01$	$1.83^{c} \pm 0.01$	$9.33^{d} \pm 0.08$	$0.58^{c} \pm 0.00$	$2.95^{c} \pm 0.01$
Protein solutions standardized with permeate								
Control	Unheated before concentration	$9.85^{f} \pm 0.00$	$6.78^{b} \pm 0.01$	$1.61^{c} \pm 0.06$	$1.04^{e} \pm 0.00$	$10.58^{a} \pm 0.01$	$0.31^{de} \pm 0.00$	$3.10^{a} \pm 0.03$
HT 10 %	Before HT	$9.89^{f} \pm 0.01$	$6.78^{b} \pm 0.01$	$1.68^{c} \pm 0.07$	$1.02^{e} \pm 0.01$	$10.30^{c} \pm 0.11$	$0.30^{de} \pm 0.01$	$3.08^{ab} \pm 0.07$
HT 20 %	Before HT	$20.66^{a} \pm 0.01$	$6.59^{f} \pm 0.01$	$3.51^{a} \pm 0.07$	$2.15^{a} \pm 0.02$	$10.41^{bc} \pm 0.10$	$0.63^{a} \pm 0.01$	$3.03^{abc} \pm 0.04$
2 HT	Before 1st HT	$9.74^{g} \pm 0.02$	$6.79^{a} \pm 0.01$	$1.55^{c} \pm 0.07$	$1.02^{e} \pm 0.00$	$10.50^{ab} \pm 0.00$	$0.30^{e} \pm 0.00$	$3.05^{abc} \pm 0.05$
	Before 2nd HT	$20.34^{c} \pm 0.15$	$6.61^{e} \pm 0.01$	$3.35^{b} \pm 0.14$	$2.11^{b} \pm 0.01$	$10.35^{bc} \pm 0.11$	$0.60^{b} \pm 0.01$	$2.97^{c} \pm 0.09$

* quantity per 100 g of sample.
Values are means ± analytical SD (n = 3). Means in a column with a common superscript letter are not significantly different ($p < 0.05$).

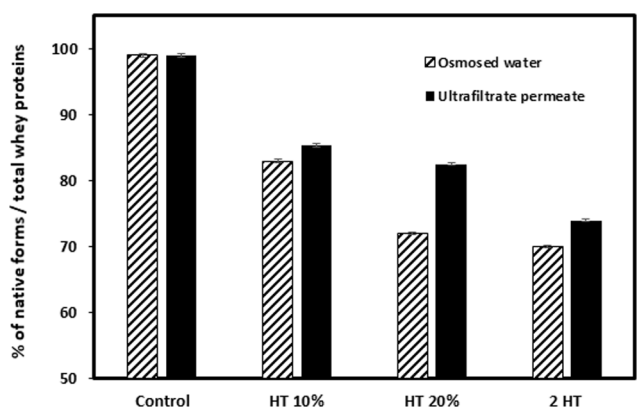

Fig. 2. Percentage of native whey proteins over total whey proteins content in the concentrated protein solutions standardized with water (▨) and with permeate (■), and produced according to the different process schemes (Control, HT 10 %, HT 20 %, 2 HT) (n = 4).

forms were observed when solutions were heat–treated. Moreover, regardless of the standardization solvent, increasing protein concentration from 10 to 20 % protein content led to more severe level of denaturation upon heating. Indeed, in water-standardized solutions, the percentage of residual native whey proteins was respectively 83 % ± 0.6 and 72 % ± 0.5 in HT 10 % and HT 20 % samples (Fig. 2). These results were consistent with previous studies reported on β–Lg (Hoffmann & Van Mil, 1997; Roefs & Kruif, 1994), on whey proteins (Farrag, Askar, El-Din, & Abd El-Salam, 1997; Tolkach, Steinle, & Kulozik, 2005; Wolz & Kulozik, 2015) and on milk protein solutions (Ho et al., 2019; Pierre, Brulé, Fauquant, & Piot, 1977). Pierre et al. (1977) heated milk protein concentrates at either 7.5 % or 17.9 % w/w protein and showed a sharp increase in the whey protein denaturation with increased protein concentrations. Ho et al. (2019) also studied milk protein concentrate solution by heat–treating either at 12 % or 17 % w/w protein and showed that whey protein aggregation was promoted at higher protein contents. Indeed, most authors suggested that increasing the protein concentration of solutions increased the probability of collision between particles by increasing Brownian motions and thus their possible interactions. We can propose here a similar phenomenon: casein micelles are rather bulky structures and at the working concentrations it is possible to have a "close packing" of the entities. In this view, whey proteins are very close to each other but also at very short distance or even interpenetrated in casein micelles. Therefore, interactions between them are greatly favored during heat treatment. The percentages of residual native whey protein were higher in protein solutions standardized with permeate than in those standardized with water (Fig. 2). Besides, the differences between water- and permeate-standardized solutions were small at 10 % protein content but noticeable at 20 % protein content (respectively 2.5 % ± 1.1 and 10.5 % ± 1.2). Some studies showed that an increase in dry matter of milk before heat treatment induced a reduction of whey protein denaturation/aggregation (Anema, 2000; McKenna & O'Sullivan, 1971). Most authors mentioned that increasing lactose content might explain this phenomenon. Indeed, lactose is known to have a protective effect on the denaturation/aggregation of whey proteins (Spiegel, 1999; Timasheff, 2002). Plock et al. (1998) proposed a protective mechanism on whey protein denaturation related to steric hindrance induced by lactose attached to soluble proteins concentrated sweet whey. In this study, it can be assumed that the lactose–proteins interactions might not be enough to have a protective effect of lactose in 10 % sample standardized with permeate. However, at 20 % protein content, since the molecules in the system are more packed, the protective effect of lactose on the denaturation induced by steric hindrance may then be effective. We could also propose a second hypothesis: an increase in ionic strength in the HT 20 % sample standardized with permeate compared to water could reduce the extent of whey protein denaturation. Nicorescu et al. (2008) showed that an increase in ionic strength from 0 to 100 mM reduced the heat-induced protein denaturation by a factor of 10. Consequently, we could suggest that at higher lactose content and ionic strength (permeate-standardized samples), the protein denaturation at higher concentration is less pronounced.

In the 2 HT process scheme, the protein solutions were heat–treated twice, at 10 % and at 20 % protein content (Fig. 1). Somewhat surprisingly, in the solution standardized with water, the percentage of residual native whey protein was not significantly different ($p < 0.05$) to the one in the solution at 20 % protein content and heat–treated once (HT 20 %), respectively 70 and 72 %. This result could be explained as

follows: the first heat treatment at 10 % protein content induced protein denaturation and led to the formation of protein aggregates. These latter might have behaved as inert materials during the second heat treatment. During the second heat treatment, the reaction was deprived of a part of its reactive material (*i.e.* native whey proteins) resulting in less collisions, thus, less aggregates formed. Same mechanism was also suggested by Christiansen et al. (2020). These results also showed that the impact of heat treatments on the extent of whey protein denaturation was not cumulative.

In order to further understand the protein behavior during heat treatment of protein solutions, the formation of the different kinds of aggregates was studied as a function of the protein content, the physico–chemical environment and the process parameters.

3.3. Influence of protein content on the co-aggregation during heat treatment

As β-Lg and α-lac are the main whey proteins, the focus was on their distribution within the different kind of states: native form, soluble aggregates or micelles-bound aggregates (Fig. 3).

In the solutions standardized with water and at a pH close to 6.75 (Table 1), regardless of the process schemes wherein heat treatment was applied, most of the denatured β-Lg were located in micelle–bound aggregates, *i.e.* from 80 % for HT 20 % to 98 % for the 2 HT process scheme (Fig. 3A). It is mainly accepted that denatured α-lac cannot initiate the aggregation process alone, and its behavior is greatly subordinated to denatured β-Lg (Wijayanti et al., 2014). Therefore, we investigated the localization of denatured α-lac in order to lend weight to the denatured β-Lg repartition. As expected, our results showed that the denatured α-lac were also mainly located in micelle-bound aggregates (Fig. 3C). Therefore, we could presume that heat treatment of solutions standardized with water, mostly induced β-Lg/α-lac mixed aggregates bound to casein micelles. In the literature, many authors investigated the formation of κ-casein/whey protein complexes and their repartition, either in the soluble phase or bound to casein micelles, during heat treatment of milk (Anema & Klostermeyer, 1997, Anema, 2009, Anema & Li, 2003, Donato & Guyomarc'h, 2009, O'Connell & Fox, 2003, Oldfield, Singh, Taylor, & Pearce, 2000, Singh, 2004, Singh & Fox, 1985, 1987, Smits & Van Brouwershaven, 1980). Heating milk at pH around 6.7–6.8 leads mainly to the formation of soluble aggregates whereas micelle bound aggregates form in majority at pH around 6.3–6.4. Anema & Li (2003) showed that during heat treatment of milk at pH 6.7, 70 % of soluble aggregates are formed. Some authors proposed that dissociation of κ-casein from the micelle at pH 6.8 or above could explain the preferred formation of κ-casein/whey protein complexes in the soluble phase (Anema & Li, 2000, Anema & Klostermeyer, 1997a,b, Donato & Guyomarc'h, 2009). Our results clearly suggest that the pathways leading to the association between κ-casein and whey proteins are modified when concentrated protein solutions with reduced levels of minerals and lactose were heat–treated. Indeed, more micellar complexes were formed at 10–20 % protein than in milk which corroborates the results observed by Christiansen et al. (2020). One of the hypotheses is that steric hindrance due to the packing of casein micelles and the short distance between casein micelles and whey proteins seems to promote their interactions. This phenomenon appears to be enhanced at ionic strength lower than that of milk.

In permeate-standardized solutions (Fig. 3B), the protein concentration between 10 and 20 % did not modify the heat-denatured β-Lg and α-lac repartition. Indeed, denatured β-Lg were located both in micelle-bound aggregates and in soluble aggregates, 55 % and 45 %, respectively. Anema & Li (2003) found 20 % micellar aggregates in heat-treated milk at pH close to 6.75 whereas we obtained for the same pH (Table 1) an equal repartition of denatured β-Lg between soluble and micelle bound aggregates. This difference could be explained by the fact that heat-treated solutions were greatly different (milk and concentrated protein solutions). In the solutions resulting from the HT 20 % and 2 HT process schemes (pH about 6.60), 60 % of the denatured β-Lg were bound to casein micelles. This result is in agreement with Anema & Li

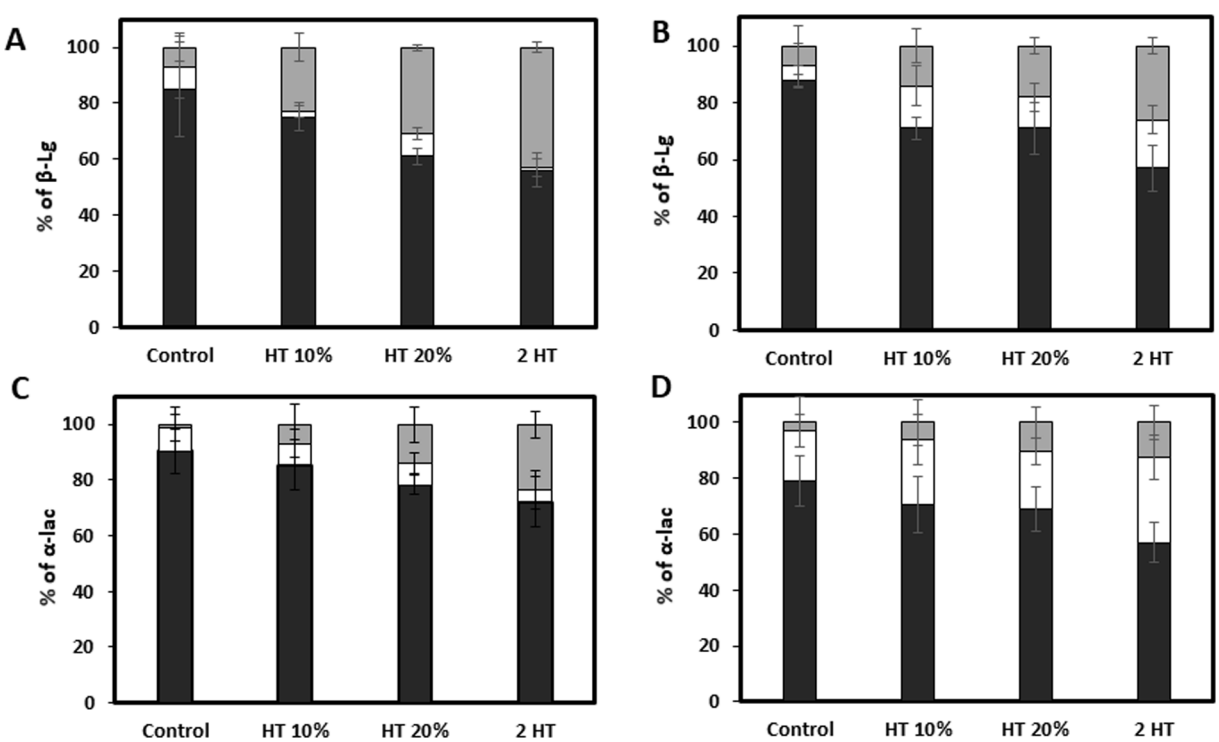

Fig. 3. Distribution of β-lactoglobulin (β-Lg) and α-lactalbumin (α-lac) as a function of their molecular state: native form (■), soluble aggregates (□), micelle bound aggregates (▩) in the protein solutions (n = 4), standardized with water (A and C) or with permeate (B and D), and produced according to the different process schemes (Control, HT 10 %, HT 20 %, 2 HT).

(2003) that found 51 % of micellar aggregates in heat–treated milk at pH 6.60.

As shown previously, the partitions of denatured β-Lg and α-lac in water-based solutions were similar (Fig. 3A and 3C, respectively). On the contrary, in permeate-based solutions, at least 75 % of the denatured α-lac whereas only 45 % of denatured β-Lg were present in soluble aggregates (Fig. 3D and 3B, respectively). Donato & Guyomarc'h (2009) proposed two pathways for the association of κ-casein/whey protein on micelles: (1) Fixation of denatured whey protein directly on the micelles without formation of primary aggregates; (2) Formation of primary aggregates in the soluble phase and then complexation of theses aggregates with micellar κ-casein. If the 2nd mechanism had occurred in the permeate-based solutions, denatured α-lac would have had a similar partition than that of denatured β-Lg. As it was not the case, we proposed that denatured β-Lg could interact indifferently and simultaneously with micellar κ-casein and with other whey proteins in the soluble phase. Moreover, in this potential pathway, association between denatured α-lac and β-Lg in the soluble phase might be favored over association on casein micelles.

3.4. Kinetics of rennet coagulation

Rennet coagulation was carried out on reconstituted solutions at 50 $g.kg^{-1}$ protein content. According to Fig. 4A and 4B, the changes in storage modulus G' during rennet coagulation can be divided in three phases: 1) lag stage from 200 to 700–900 s of casein hydrolysis; 2) coagulation stage from 700 to 900 to 2700 s, characterized by a strong increasing rate in G' due to the interactions between micelles, incorporation of new casein materials into the gel network and to a spatial reorganization of micelles and network; 3) slowdown of G' rise from 2700 to 3600 s. For protein solutions standardized with water (Fig. 4A), the heat treatment of HT 10 % solution induced a slight reduction of the gel firming rate at the beginning of the process, between 500 and 1500 s, compared to the unheated solution. Nevertheless, resulting gels of both HT 10 % and unheated solutions reached the same G' at 3000 s. The sample resulting from the HT 20 % process scheme showed a delay in the increase of the storage modulus while the 2 HT sample had the same gel time as the control sample. However, both HT 20 % and 2 HT samples displayed the same evolution of G' from 1300 to 1500 s with lower firming rate and final G' than the control sample (Fig. 4A). For the protein solutions standardized with permeate, the control sample had the earliest increase of G' and the highest gel firming rate whereas the 2 HT sample had the highest delay in the increase of G' and the lowest gel firming rate. Finally, the HT 10 % and HT 20 % samples showed a quite similar behavior. They had intermediate values for the delay in the increase of G' and the gel firming rate compare to the control and 2 HT samples (Fig. 4B).

During coagulation of protein formulas, tan δ decreased down to a plateau at about 2000 s. Two groups of samples could be identified for both standardization solvents (Fig. 4C and 4D). Indeed, the control and HT 10 % samples showed the highest final tan δ, whereas HT 20 % and 2 HT gels had a lower tan δ value. Moreover, whereas tan δ slightly increased after its minimum in the control and HT 10 %, it was constant for HT 20 % and 2 HT gels.

3.5. Coagulation properties

Milk protein coagulation can be described as a three–step process: hydrolysis of κ–casein, aggregation of *para*-micelles and gel firming. These steps can occur simultaneously during the coagulation (Britten & Giroux, 2022). In order to have a better understanding of the impact of heat treatment and physico-chemical environment on the coagulation properties of the samples, some key parameters were extracted from the coagulation curves (Fig. 4, Table S1) and analysed using a PCA (Fig. 5).

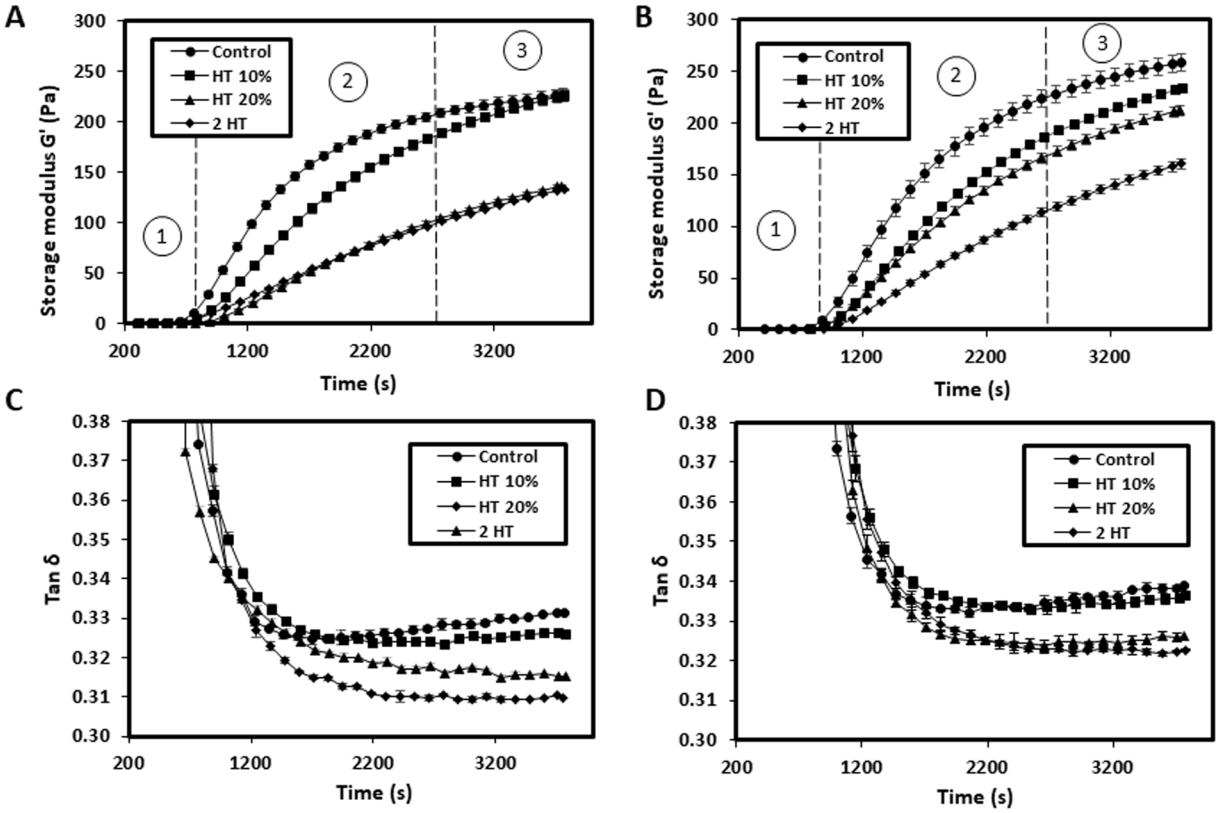

Fig. 4. Evolution of the storage modulus G' and loss tangent tan δ of reconstituted solutions at 50 $g.kg^{-1}$ protein. Powders used for reconstitution were produced according to different process schemes (Control, HT 10 %, HT 20 % and 2 HT) from protein solutions standardized with water (A and C) or permeate (B and D) (n = 6). 1) lag stage; 2) strong increase of G'; 3) slowdown of G' rise.

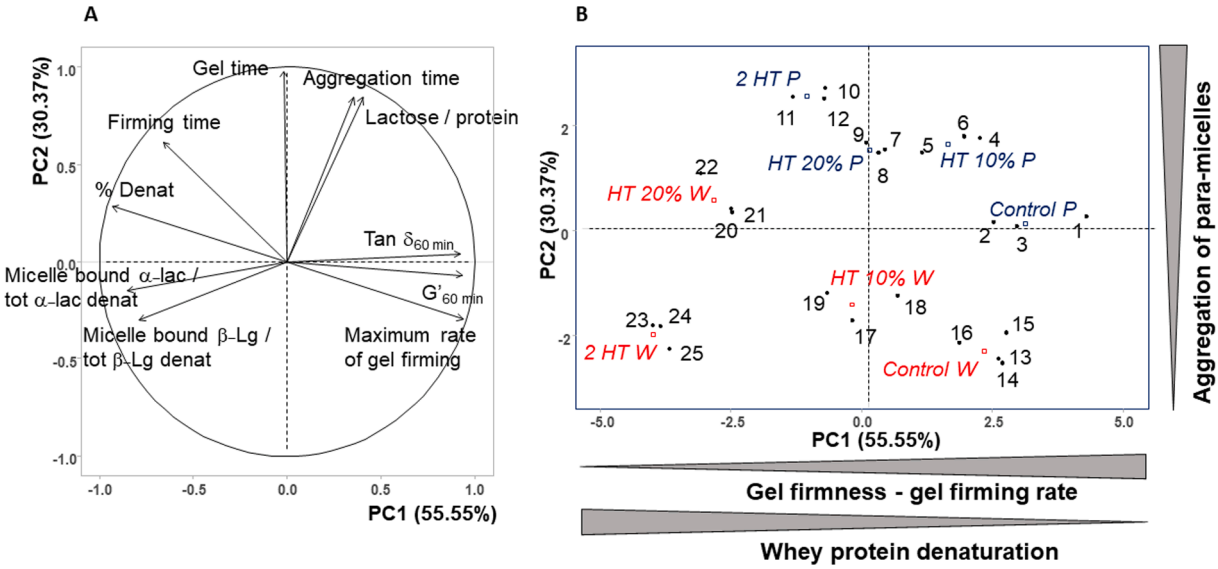

Fig. 5. PC1-PC2 factorial map. (A): Correlation circle of the PCA performed on the coagulation parameters obtained from aggregation and coagulation curves. Micelle bound β-Lg / tot β-Lg denat and micelle bound α-lac / tot α-lac denat correspond to the amount of denatured protein bound to the casein micelles over the total amount of denatured whey protein. % Denat: whey protein denaturation level. The correlations between the variables are shown by the angle between their projections in the circle. (B): Projection of individuals in the factorial map following their similarities, according to the different process schemes (*Control, HT 10%, HT 20% and 2 HT*) from protein solutions standardized with water (*W*) or permeate (*P*).

PCA provides an "objective" summary of the data with a controlled loss of information. In the present work, the first (PC1) and the second (PC2) principal components accounted for respectively 55.55 % and 30.37 % of the total variance. All the other principal components each accounted for <8 % of the total variance. In the following, only PC1 and PC2 were therefore be considered. The variables associated with PC1 and PC2 are shown in Fig. 5A. The graph of corresponding individuals where proximity reflects similarity is presented in Fig. 5B. The PCA suggested a representation of the variables into three distinct groups: (1) first group mainly including variables that correspond to whey protein denaturation; (2) second one including variables related to the rheological characteristics of the gels and (3) third one with variables related to the kinetics of aggregation of *para*-micelles and a variable related to the lactose/protein ratio. The first group was negatively correlated to PC1, the second one was positively correlated to PC1, whereas the third one was positively correlated to PC2 (Fig. 5A). Thereby, rheological properties of gels were exclusively determined by the protein denaturation intensity, whereas the kinetics of aggregation were correlated to the nature of the aqueous phase. It follows that rheological properties were decorrelated from the kinetics of the early gel formation. Two clusters of samples were opposed on PC1: (1) unheated samples and (2) samples that were heat-treated twice (Fig. 5B). The samples were evenly distributed along PC2: permeate–standardized samples on the top right and water–standardized ones on the bottom left. As a rule of interpretation and for example, since PC1 was highly positively correlated with $G'_{60\ min}$ and negatively correlated with "%denat" (Fig. 5A), any sample with high positive coordinates on PC1 were then be characterized by a high $G'_{60\ min}$ and a low protein denaturation percentage (Fig. 5B). Similarly, since PC2 was highly positively correlated with gel time and lactose/protein ratio (Fig. 5A), any sample with high positive coordinates on PC2 were then be characterized by a high gel time and lactose/protein ratio (Fig. 5B).

As shown in Fig. 5A (correlation matrix in supplementary data: Figure S1), the aggregation time was strongly correlated with the lactose/protein ratio ($R^2 = 0.73$, $p < 0.001$): the permeate samples had a longer *para*-casein micelles (*para*-CMs) aggregation time than the water samples. The higher ionic strength in these samples explained this delay as an increase in ionic strength slows down the hydrolysis of k-casein by chymosin (Bringe & Kinsella, 1986; Famelart, Le Graet, & Raulot, 1999; Visser, Rooijien, & Slangen, 1980). Moreover, the higher the ionic strength, the higher the ionization of the weak acids of proteins and the higher negative charge of the micelle. This could increase electrostatic repulsions between them and therefore the association time between the renneted-micelles. Indeed, Dalgleish (1983) suggested that the aggregation of renneted–micelles were due to the formation of ion-pairs between them and these specific surface interactions would be inhibited by increasing the ionic strength. The differences in aggregation time between permeate– and water–standardized samples were mainly due to the physicochemical differences between the solutions, as the variables "*para*-CMs aggregation time" and "whey protein denaturation" were not correlated with each other (R^2 = -0.09). This is in agreement with Anema, Kim Lee, & Klostermeyer (2007) and Kethireddipalli, Hill, & Dalgleish (2011) who showed that the presence of whey proteins on the micelle did not interfere with the hydrolysis of κ-casein. Donato & Guyomarc'h (2009) and Singh & Waungana (2001) suggested that denatured whey proteins were likely to interfere with *para*-CMs fusion rather than on their enzymatic hydrolysis or aggregation. On the contrary, Anema, Lee, & Klostermeyer (2011) proposed that micelles with less bound whey protein complexes aggregated more rapidly. As shown in Fig. 5A, aggregation time and gel time were highly correlated (R^2 = 0.87, $p < 0.001$). The 2 HT water-based sample showed a singular behavior, which deserves to be discusses (Fig. 5). One might expect that the aggregation time would be similar to that of the control since they have the same gel time. Yet it has the lowest aggregation time (negative score on PC1 and PC2) and the same gel time as its control (same score on PC2). It could be presumed that the successive heat treatments in the process scheme with water as standardization phase could induce physico-chemical environment changes that might partly lead to the screening of the negative charges of casein micelles. Therefore, micelles hydrolysis and their association would be promoted in a more effective way in the 2 HT sample than in the other samples. We could also suggest that the casein micelles structure was modified during the 2 HT process scheme leading to a promotion of their interaction. Further analysis is needed to have a better understanding of this phenomenon.

Aggregation time and gel time both showed the same correlation behavior in regard to the lactose/protein ratio and the level of whey protein denaturation. Our study suggested then that the gel time and the protein denaturation were not significantly correlated (R^2 = 0.29). This

result was in agreement with Giroux, Lanouette, & Britten (2015) who found no significant effect of an increase of the extent of whey protein denaturation on the gel time. Waungana et al. (1996) showed that denaturation of β-Lg up to 60 % had no effect on the gelation time, but above this value gelation time increased. On the contrary, Giroux et al. (2020) found a correlation between these latter even at low protein denaturation level. It could be explained by differences of protein concentration (3.3 *vs* 5 % w/w), physico-chemical environment (milk *vs* pure protein system), and heating process.

As presented in Fig. 5A, the aggregation time of the *para*-micelles was not correlated with the firming kinetics of the gel as well as its final rheological properties. This suggested than the kinetics of κ-casein hydrolysis and of renneted-micelles association did not influence the rate of spatial reorganization of *para*-CMs and their fusion along with finals G' and tan δ. The level of denaturation was highly negatively correlated ($p < 0.001$) with these latter (R^2 = -0.89: G'; −0.86: tan δ; −0.99: maximum rate of gel firming). We assumed here that at high protein concentration and temperature about 74 °C, all denatured protein associated into protein aggregates. Thereby, these results suggest that the disruption of the gel reorganization was mainly linked to the presence of heat-induced protein aggregates in the solutions. Indeed, control samples (without protein aggregates) had a higher G', tan δ and firming rate compared to heat-treated samples (Fig. 5B). Our study is in agreement with Giroux et al (2020, 2015), Perreault, Morin, Pouliot, & Britten (2017) that proposed that whey protein/caseins complexes, either in the soluble phase or on the micelles, disrupted the spatial reorganization of the *para*-CMs and *para*-CMs clusters by steric hindrance and then disturbed the gel firming. As displayed in Fig. 5A, the maximum rate of gel firming was perfectly correlated to the level of whey protein denaturation (R^2 = -0.99, $p < 0.001$). Thereby, the impact of the protein denaturation/aggregates content could also be expressed as the modification of the maximum rate of rearrangement of *para*-CMs clusters and incorporation of free *para*-CMs within the gel.

Moreover, the tan δ and the storage modulus were positively correlated ($p < 0.001$) (Fig. 5A). Gels with higher G' (control samples) showed more viscous characteristics, *i.e.* are more prone to flow. As presented before, an increase in whey protein denaturation induced weaker gels but with higher elastic properties. The presence of whey protein/κ-casein complexes in the solutions led to longer relaxation time bonds between casein micelles. Preheating milk is known to lead to rennet gels with thicker protein cross-links and more rigid network that are less prone to flow than with unheated milk (Miloradovic et al., 2020). Indeed, the rearrangement of the gel depends on the adhesive forces between micelles and their viscoelastic properties *i.e.* their ability to behave "liquid–like" (Mellema, Walstra, Van Opheusden, & Van Vliet, 2002). The presence of protein aggregates could prevent an optimal reorganization of the gel and then lead to a weaker gel. This phenomenon seemed to be enhanced when micelle-bound aggregates were dominantly present (Fig. 5A). Whey protein aggregates could interact with each other and then increased the solid–like properties of the protein network. Similar phenomenon has already been observed between aggregates at the surface of oil droplets (Loiseleux et al, 2018). However, the correlation between the percentage of micelle-bound whey protein and the tan δ was lower compared to the correlation between the latter and the level of whey protein denaturation (R^2 = -0.64 and -0.86, respectively).

As shown in Fig. 5A, the level of whey protein denaturation was positively correlated to the micelle-bound aggregate fraction (R^2 = 0.62 for β-Lg and 0.70 for α-lac). In order to separate these two phenomena and better investigate the effect of protein aggregates partition, samples with a similar amount of protein aggregates but a different aggregates partition were compared (Table 2). Thereby, differences in gel time and $G'_{60\ min}$ over control samples were discussed. An increase in the micelle-bound to total aggregates ratio led to a higher gel time delay (Table 2) as already suggested by Vasbinder & De Kruif (2003). This is mainly due to an increase of the delay between the aggregation and the early gelation (G' > 1 Pa) explained by a steric hindrance effect, reducing the firming rate. However, the impact of the localization of the aggregates was only significant ($p < 0.05$) at relatively high protein denaturation level obtained in our study, *i.e.* at about 27 %. It could be proposed that the steric hindrance effect was more effective when a higher number of casein micelles were coated by whey protein aggregates or when the coating of micelles was more intense. This result could also be explained by a difference in the homogeneity of the whey protein coating as proposed by Vasbinder & De Kruif (2003). As displayed in Table 2, an increase in the percentage of micelle-bound aggregates fraction induced a lower $\Delta G'_{60\ min}$. The HT 10 % water-based sample (HT 10 % W) contained protein aggregates but it had the same final gel firmness as the unheated sample. On the contrary, in the HT 10 % permeate–based sample, we observed an increase of the $\Delta G'_{60\ min}$ even if the latter contained only a low amount of aggregates. This result was in agreement with Giroux et al. (2020) who found a significant increase in $\Delta G'_{60\ min}$ even with 4 % of protein denaturation. Thereby, in the HT 10 % W sample, it seemed than the presence of micelle-bound aggregates was insufficient to disrupt irreversibly gel reorganization even if the coagulation kinetics were modified as shown in Fig. 4. However, the impact of location was only significant ($p < 0.05$) at low protein denaturation level (about 17 %). Indeed, at higher denatured protein content (about 27 %), the final gel firmness was reduced regardless of the location of aggregates which is in agreement with results found with heated milk (Anema, Kim Lee, & Klostermeyer, 2007, Giroux et al., 2020; Kethireddipalli, Hill, & Dalgleish, 2010).

3.6. Phenomena acting on coagulation properties

Based on the results of the study, gel time and gel firmness in function of technological routes for both standardizing solutions (water (Fig. 6A) and permeate (Fig. 6B)) were presented. A schematic presentation of protein denaturation level and the spatial distribution of whey protein and casein micelles in the concentrated solutions is also proposed (Fig. 6). Heat treatment of solutions standardized with water led to the formation of micellar aggregates. HT 20 % and 2 HT contained

Table 2
Impact of whey protein aggregates partition on gel time and gel firmness.

Sample	Standardization based	Denaturation level (%)	β-Lg micelles[1] (%)	α-lac micelles[1] (%)	Gel time (s)	Gel time delay (s)	$G'_{60\ min}$ (Pa)	$\Delta G'_{60\ min}$ (Pa)
Control	Water	–	–	–	$643^e \pm 6$	–	$227^b \pm 6$	–
Control	Permeate	–	–	–	$775^c \pm 16$	–	$258^a \pm 8$	–
HT 10 %	Water	$17.0^c \pm 0,5$	$92.0^a \pm 7$	$47.2^b \pm 30$	$717^d \pm 17$	$74^c \pm 17$	$227^b \pm 5$	$0^a \pm 5$
HT 20 %	Permeate	$17.5^c \pm 0,4$	$62.1^c \pm 4$	$32.9^b \pm 13$	$839^b \pm 8$	$64^c \pm 8$	$212^c \pm 5$	$-46^b \pm 5$
HT 20 %	Water	$28^a \pm 0,9$	$79.5^b \pm 7$	$65.1^a \pm 18$	$829^b \pm 9$	$186^a \pm 8$	$138^e \pm 2$	$-89^c \pm 3$
2HT	Permeate	$26^b \pm 0,7$	$60.5^c \pm 2$	$28.9^b \pm 16$	$894^a \pm 7$	$119^b \pm 7$	$160^d \pm 5$	$-98^c \pm 5$

1: β-Lg micelles and α-lac micelles refer to the amount of denatured protein bound to the casein micelles over the total amount of denatured whey protein.
Values are means ± analytical SD (n = 3). Means in a column with a common superscript letter are not significantly different ($p < 0.05$).

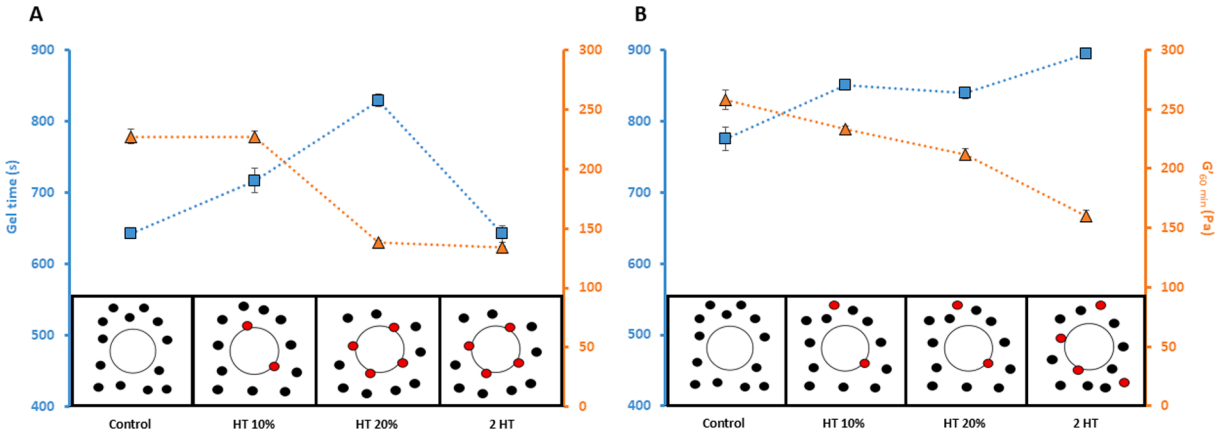

Fig. 6. Gel time (■, y–axis on the left), gel firmness G' $_{60\ min}$ (▲, y–axis on the right), schematic presentation of measured protein denaturation/(co–) aggregation level extracted from Fig. 3 (casein micelle ◯, whey protein in native form ●, whey protein aggregates ●), according to different process schemes (Control, HT 10 %, HT 20 % and 2 HT) from protein solutions standardized with water (A) and permeate (B) (n = 3). Lines are guides for the eye.

higher amount of protein aggregates than HT 10 %, which shows the importance of protein concentration in the denaturation/aggregation process of whey proteins. The impact of heat treatments on the extent of whey protein denaturation was not cumulative (Fig. 6A). Heat treatment of permeate-standardized solutions led to the formation of both micellar and soluble aggregates (Fig. 6B). In contrast to water-standardized samples, HT 10 % and HT 20 % showed similar aggregates contents (Fig. 6A and 6B). The increased proximity between lactose and proteins in HT 20 % might protect whey proteins from heat-induced denaturation and explain these results. As shown in Fig. 6A and 6B, there is no correlation between the whey protein denaturation and the gel time. However, putting aside the 2 HT water-standardized sample, heat-treated samples had a higher gel time than control samples. An increase in the protein aggregates content in the solutions induced a decrease in the gel firmness G'$_{60\ min}$ (Fig. 6A and 6B). However, a threshold effect was observed in water-standardized samples, as the gel firmness was not significantly different between control and HT 10 % samples (Fig. 6A).

4. Conclusions

This study aimed to demonstrate how the placement of heat treatment of concentrated milk protein systems might affect their enzymatic coagulation properties. We studied the influence of protein concentration on the denaturation/aggregation process of milk proteins in pure and highly concentrated protein system up to 20 % w/w. The influence of the physico-chemical environment of solutions during heat treatment, mainly lactose content, was also investigated.

In water-based samples, the increase in protein concentration induced higher protein aggregate contents. However, the solution heat-treated twice at 10 and 20 % w/w protein showed similar protein denaturation as the solution heat-treated once at 20 % w/w protein. On the contrary, in permeate-based samples, the increase in protein concentration did not significantly modify the final protein aggregates content in the solution. Indeed, the highly packed molecules system represented in the 20 % w/w protein solution enhanced the proposed lactose-protecting effect on heat-induced whey proteins denaturation. To the authors' knowledge, this study provides for the first time the distribution of molecular states of whey proteins and casein micelles (co-aggregation) during heat treatment of concentrated protein systems, standardized in either water or permeate. Micellar-bound aggregates were mostly formed in water-based solutions whereas both soluble and micellar-bound aggregates were produced in permeate-based solutions. These results, in particularly those relative to the water-based solutions, were different to the literature dealing with heated milk at the same pH values. This work suggests that the mechanisms of association between whey protein and casein micelles were modified in concentrated systems. We also showed that the association between β-Lg and α-lac seemed to be privileged in the soluble phase in permeate-based solutions. It puts aside one of the pathways proposed by Donato & Guyomarc'h (2009) where primary aggregates were first formed in the soluble phase and then transferred to casein micelles. Finally, this work demonstrated that rheological properties of gels were exclusively determined by the protein denaturation intensity, whereas the kinetics of aggregation was correlated to the nature of the aqueous phase. Moreover, the impact of the localization of protein aggregates, soluble or micellar, was different at "low" or "high" aggregates content. Some specificities were observed for water–standardized samples, particularly the gel firmness for HT 10 % and the aggregation time for 2 HT. Further investigations are required to better understand phenomena taking place in these samples.

This work provides new scientific knowledge about the mechanisms of protein denaturation/aggregation occurring in concentrated protein systems. It is also relevant for industrial applications relative to the manufacture and the use of high protein powders. It demonstrated the impact of the process scheme on the physico-chemical characteristics of powders (protein denaturation, spatial distribution of protein aggregates) and on their resulting functionalities.

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CRediT authorship contribution statement

François Martin: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Jeehyun Lee:** Conceptualization, Writing – original draft, Writing – review & editing, Visualization. **Luisa Azevedo-Scudeller:** Methodology, Formal analysis, Investigation, Writing – review & editing. **Arnaud Paul:** Writing – review & editing. **Guillaume Delaplace:** Writing – review & editing, Funding acquisition. **Jennifer Burgain:** Writing – review & editing, Funding acquisition. **Florence Rousseau:** Methodology, Investigation, Writing – review & editing. **Gaëlle Tanguy:** Conceptualization, Writing – review & editing, Visualization, Supervision. **Marie-Hélène Famelart:** Methodology, Writing – review &

editing. **Romain Jeantet:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Cécile Le Floch-Fouéré:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.112030.

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